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㉔ Antibody heteroconjugates for the killing of HIV-infected cells.

㉔ The present invention relates to novel antibody heteroconjugates and their use in methods for killing HIV-infected cells. The heteroconjugates are comprised of an antibody specific for an HIV antigen that is expressed on HIV-infected cells cross-linked to an antibody specific for an effector cell of the peripheral blood capable of killing an HIV-infected target cell. The antibody heteroconjugates of the invention physically bridge the effector cell to the target cell and may activate the lytic mechanism of the effector cell in the killing of the HIV-infected target cell. The methods, heteroconjugates, pharmaceutical compositions and combinations described herein provide a novel approach to the treatment of HIV-infected individuals by amplifying endogenous HIV-specific effector mechanisms and may also be of prophylactic value in individuals newly infected or accidentally exposed to the HIV virus.

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ANTIBODY HETEROCONJUGATES FOR THE KILLING OF HIV-INFECTED CELLS

TECHNICAL FIELD OF THE INVENTION

5 The present invention relates to novel antibody heteroconjugates and their use in methods for killing cells infected with Human Immunodeficiency Virus (HIV) in the treatment of HIV infections. More particularly, the invention relates to the construction of antibody heteroconjugates comprising an antibody specific for a particular peripheral blood effector cell cross-linked to an antibody specific for an HIV antigen present on the surface of HIV-infected cells. Such antibody heteroconjugates physically bridge the effector cell to the target cell to be killed and may activate the lytic mechanism of the effector cell in the killing of the HIV-infected target cell. The antibody heteroconjugates and methods of this invention provide a novel approach to the treatment of HIV-infected individuals by amplifying endogenous HIV-specific effector mechanisms and may also be of prophylactic value, before the development of HIV immune responses, in individuals newly infected or accidentally exposed to the HIV virus.

BACKGROUND OF THE INVENTION

20 The infectious agents responsible for acquired immunodeficiency syndrome (AIDS) and its prodromal phases, AIDS-related complex (ARC) and lymphadenopathy syndrome (LAS), are novel lymphotropic retroviruses recently termed Human Immunodeficiency Virus (HIV 1 and 2). Isolates of these viruses include LAV-1, LAV-2, HTLV-III, and ARV.

The general structure of HIV is that of a ribonucleoprotein core surrounded by a lipid-containing envelope which the virus acquires during the course of budding from the membrane of the infected host cell. Embedded within the envelope and projecting outward are the viral-encoded glycoproteins. For example, the envelope glycoproteins of HIV-1 are initially synthesized in the infected cell as a precursor molecule of 150,000-160,000 daltons (gp150 or gp160), which is then processed in the cell into an N-terminal fragment of 110,000-120,000 daltons (known in the art as gp110 or gp120) to generate the external glycoprotein, and a C-terminal fragment of 41,000-46,000 daltons (gp41), which represents the transmembrane envelope glycoprotein. The internal viral proteins of HIV include the "gag" and "pol" proteins.

As the spread of HIV reaches pandemic proportions, the treatment of infected individuals and prevention of the virus' transmission to uninfected individuals at risk of exposure is of paramount concern. A variety of therapeutic strategies have targeted different stages in the life cycle of the virus and are outlined in Mitsuya and Broder, *Nature*, 325, p. 773 (1987). One approach involves the use of antibodies specific for antigens on the HIV glycoproteins. These antibodies may inhibit viral replication, either by interfering with viral entry into host cells or by some other mechanism. Once the viral proteins or the antigenic determinants on those proteins that are susceptible to antibody intervention are identified, antibody titers sufficient to neutralize the infectivity of the virus could be engendered by vaccination or, alternatively, by the passive administration of immunoglobulins or monoclonal antibodies of the desired antigenic specificity.

The gp110 glycoprotein of HIV-1 has been the object of much investigation as a potential target for interfering with the virus' infectivity. Sera from HIV-infected individuals have been shown to neutralize HIV *in vitro* and antibodies that bind to purified gp110 are present in the sera [see M. Robert-Guroff et al., *Nature*, 316, pp. 72-74 (1985); R.A. Weiss et al., *Nature*, 316, pp. 69-72 (1985); and Mathews et al., *Proc. Natl. Acad. Sci. U.S.A.*, 83, p. 9709 (1986)]. Purified and recombinant gp110 have stimulated the production of neutralizing serum antibodies when used to immunize animals [see Robey et al., *Proc. Natl. Acad. Sci. U.S.A.*, 83, p. 7023 (1986) and Lasky et al., *Science*, 233, p. 209 (1986)]. Immunization of a human with a recombinant vaccinia virus that expresses HIV gp110 and gp41 induced HIV-neutralizing antibodies [see Zagury et al., *Nature*, 326, p. 249 (1986)]. Binding of the gp110 molecule to the CD4 (T4) receptor has also been shown and monoclonal antibodies which recognize certain epitopes of the CD4 receptor have been shown to block HIV binding, syncytia formation and infectivity [see McDougal et al., *Science*, 231, p. 382 (1986)]. Putney et al., *Science*, 234, p. 1392 (1986) elicited neutralizing serum antibodies in animals after immunizing with a recombinant fusion protein containing the carboxyl-terminal half of the gp110 molecule and further demonstrated that glycosylation of the envelope protein is unnecessary for a neutralizing antibody response. Furthermore, monoclonal antibodies to HIV glycoproteins such as gp110 and gp41 have

activation of effector cells such as peripheral blood lymphocytes and administration of the activated effector cells and the heteroconjugates to the HIV-infected patient.

The methods, heteroconjugates, pharmaceutical compositions and combinations of this invention are useful for killing HIV-infected cells in individuals suffering from HIV infections and may be particularly useful if the heteroconjugates contain one or more antibodies that neutralize HIV infectivity or if the heteroconjugates are administered together with HIV-neutralizing antibodies. In addition, these methods and heteroconjugates may be of prophylactic value in the treatment of individuals newly or accidentally infected with HIV.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a comparative graphical presentation of the % lysis of HIV-infected cells by anti-CD3-pretreated or untreated PBL from seronegative donors vs. antibody concentration of either the 110.4 x G19-4 heteroconjugate of one embodiment of this invention or a control mixture of the individual antibodies that make up the heteroconjugate.

Figure 2A depicts in table form the % lysis of HIV-infected and uninfected cells by anti-CD3-pretreated PBL from seropositive and seronegative donors in the presence of either the 110.4 x G19-4 heteroconjugate of one embodiment of the invention or a control mixture of the individual antibodies that make up the heteroconjugate (NT = not tested).

Figure 2B depicts in table form the % of HIV-infected cells by anti-CD3-pretreated unseparated or CD8⁺-enriched PBL from seronegative donors in the presence of the 110.4 x G19-4 heteroconjugate of one embodiment of this invention.

Figure 3 depicts a comparative graphical presentation of the % lysis of HIV-infected cells by untreated or (A) interleukin-2 (IL-2)-pretreated or (B) β -interferon (β -IFN)-pretreated PBL from seronegative donors vs. antibody concentration of either the 110.4 x Fc2 heteroconjugate of one embodiment of this invention or a control mixture of the individual antibodies that make up the heteroconjugate.

Figure 4 depicts in table form the % lysis of HIV-infected cells by PBL from seronegative donors, that were pretreated for 3 hours with varying concentrations of β -IFN, in the presence of the 110.4 x Fc2 heteroconjugate of one embodiment of the invention, a mixture of the individual antibodies that make up the heteroconjugate, each individual antibody of the conjugate alone or in the absence of any heteroconjugate or antibodies.

Figure 5 depicts in table form the % lysis of HIV-infected and uninfected cells by IL-2-pretreated seropositive and seronegative PBL in the presence of either the 110.4 x Fc2 heteroconjugate of one embodiment of this invention or a control mixture of the individual antibodies that make up the heteroconjugate.

Figure 6 depicts a comparative graphical presentation of the % lysis of HIV-infected cells by CD16⁺-enriched seronegative PBL pretreated with IL-2, unseparated seronegative PBL pretreated with IL-2, and untreated unseparated seronegative PBL, in the presence of the 110.4 x Fc2 heteroconjugate over a range of effector:target (E:T) cell ratios.

Figure 7 depicts in table form the % lysis of HIV-infected cells by PBL from seronegative donors in the presence of varying concentrations of either the 41.1 x G19-4 heteroconjugate of one embodiment of the invention, a mixture of the individual antibodies that make up the heteroconjugate or each individual antibody of the heteroconjugate alone.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

The present invention relates to novel antibody heteroconjugates and their use in methods for killing HIV-infected cells. More particularly, the invention relates to heteroconjugates comprised of at least two antibodies cross-linked to each other. One antibody is specific for and reactive with an HIV antigen expressed on HIV-infected cells. The other antibody is specific for and reactive with an antigen found on effector cells of the peripheral blood capable of killing an HIV-infected target cell. Such effector cells may include cytotoxic T cells, monocytes (or macrophages), granulocytes, and LGLs, which include cells with

natural killer (NK) cell activity or ADCC activity. Since the HIV-specific antibody of the heteroconjugate binds to HIV-infected cells and the effector cell-specific antibody of the heteroconjugate binds to the cytotoxic effector cell, the heteroconjugate of this invention provides a means to bridge the two cells, bringing the cytotoxic effector cell in contact with the infected target cell and thus promoting lysis of the target cell.

Without being bound by theory, it is believed that the heteroconjugates described herein act not only to bridge cytotoxic effector cells to the HIV-infected target cells but also activate the lytic mechanisms of the effector cells [see, e.g., M.A. Liu et al., *Proc. Natl. Acad. Sci. U.S.A.*, 82, pp. 8648-8652 (1985) and P. Perez et al., *J. Exp. Med.*, 163, pp. 166-178 (1986)]. Thus, although an effector cell such as a T cell may have its own antigenic specificity, it can be retargeted by the interaction with the heteroconjugate of this invention to kill HIV-infected cells bound by the heteroconjugate. The heteroconjugate approach of this invention, therefore, provides for an enhanced HIV-specific effector cell response in HIV-infected individuals by activating effector cells such as cytotoxic T cells or LGLs and then bringing them into close proximity, via the antibody heteroconjugate bridge, with the HIV-infected target cells to be killed. In addition, such heteroconjugates can render effector cells from individuals who have not yet developed anti-HIV immunity (e.g., newly infected individuals) cytotoxic because naive effector cells can be targeted by the heteroconjugates of this invention to kill HIV-infected cells.

The antibodies that comprise the heteroconjugates of this invention may be polyclonal or preferably, monoclonal. The term "antibody" as used in this application includes intact antibody molecules or Fab or F-(ab)₂ fragments. If monoclonal antibodies are used, the antibodies may be of mouse or human origin or chimeric antibodies. The antibodies that comprise the heteroconjugates of this invention can be covalently bound to each other by techniques well known in the art such as the use of the heterobifunctional cross-linking reagent SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate) [see e.g., B. Karpovsky et al., *supra*]. Alternatively, the antibodies may be covalently bound to each other using GMBS (maleimido butyloxysuccinimide) as described by R.R. Hardy, in *Methods Immunol.*, 4th Ed., D.M. Weir (ed.), pp. 31.1-31.12 (1986). Furthermore, an embodiment of the invention may involve heteroconjugates comprised of more than two antibodies. For example, a heteroconjugate of the invention may be comprised of two antibodies specific for the effector cell and one antibody specific for the target HIV-infected cell. Alternatively, the heteroconjugate may be comprised of two antibodies specific for the target cell and one antibody specific for the effector cell. These heteroconjugates are covalently bound to each other by techniques known in the art as cited above.

The HIV-specific antibody of the heteroconjugate may be any antibody that is specific for and reactive with an HIV antigen that is sufficiently exposed or expressed on the surface of HIV-infected cells. The antibody should, additionally, have an affinity for the HIV antigen on the cell surface such that the antibody heteroconjugate forms a stable bridge between the infected target cell and the effector cell. The antibody should preferably have an affinity association constant on the order of about 10⁻⁸ to about 10⁻¹².

The effector-specific antibody of the heteroconjugate may be any antibody that is specific for and reactive with an effector cell of the peripheral blood capable of killing an HIV-infected cell. Preferably, the antibody is one that reacts with an antigen on the surface of the effector cell such that the lytic mechanism of the effector cell is activated. Such antibodies may include antibodies that react with epitopes on T lymphocytes such as CD3 [see S.C. Meuer et al., *J. Exp. Med.*, 157, p. 705 (1983)], CD28 (also known in the art as Tp44) [see J.A. Ledbetter et al., *J. Immunol.*, 137, pp. 3299-3305 (1986) and *Leukocyte Typing III*, A.J. McMichael (ed.), Oxford University Press, Oxford (in press)], and CD2 [see C.H. June et al., *J. Clin. Invest.*, 77, p. 1224 (1986) and *Leukocyte Typing*, A. Bernard et al. (ed.s), Springer-Verlag, New York (1984)]. Alternatively, the effector-specific antibody component of the heteroconjugate may include antibodies that react with epitopes on the Fc receptors of certain effector cells such as LGLs, granulocytes or monocytes. Examples of such antibodies include antibodies specific for the CD3/T cell receptor complex on T lymphocytes such as the G19-4 antibody [see, e.g., J.A. Ledbetter et al., *J. Immunol.*, 135, pp. 2331-2336 (1985)] and antibodies that react with the CD16 Fc receptor of LGLs such as the Fc2 antibody [see, e.g., J.A. Ledbetter et al., in *Perspectives In Immunogenetics And Histocompatibility*, Vol. 6, E. Heise (ed.), Lymphocyte Surface Antigens 1984, pp. 325-340, American Society For Histocompatibility And Immunogenetics, New York (1984)]. Both anti-CD3 and anti-CD16 monoclonal antibodies are commercially available (e.g., Leu 4 and Leu 11 antibodies, respectively, Becton Dickinson, Mountainview, CA).

According to one preferred embodiment of this invention, a monoclonal antibody to the HIV-1 glycoprotein gp110 (110.4) was cross-linked to a monoclonal antibody to the CD3 antigen found on the T cell receptor (G19-4). The heteroconjugate targeted T cells from PBL of seropositive and seronegative humans to kill HIV-infected cells.

According to our experimental protocol, PBL were incubated with radiolabeled chromium (⁵¹Cr) HIV-

infected target cells in the presence of a 110.4 x G19-4 heteroconjugate of this invention and lysis of the target cells determined by the release of the ^{51}Cr label into the medium. We found that the PBL lysed the HIV-infected cells in the presence of the heteroconjugate whereas little or no lysis occurred in the presence of a mere mixture of the individual monoclonal antibodies that made up the conjugate.

In addition, we found that PBL preincubated with anti-CD3 and then exposed to the target cells in the presence of the 110.4 x G19-4 heteroconjugate were even more cytotoxic to the HIV-infected cells than untreated PBL. This result is in agreement with studies directed to the use of heteroconjugates against tumor cells which reported that pretreatment with antibody to the T3 antigen on T cells stimulates or augments the lytic mechanism of the T cell [see, e.g., G. Jung et al., *supra*].

Of great importance was the fact that PBL from asymptomatic HIV-infected, i.e., seropositive individuals, were also capable of lysing HIV-infected cells in the presence of the 110.4 x G19-4 heteroconjugate. Thus, the heteroconjugates and methods of this invention may provide a means for enabling or augmenting the ability of effector cells in the blood of individuals already infected with HIV to kill HIV-infected cells.

Furthermore, we found that anti-CD3-activated PBL enriched for CD8⁺ cells (CD8 being an antigenic marker for cytotoxic T cells) were more cytotoxic than unfractionated PBL. This observation suggests that CD8⁺ cytotoxic T cells within the PBL population are targeted by the 110.4 x G19-4 heteroconjugate for the lysis of the HIV-infected cells.

In a second preferred embodiment, a monoclonal antibody to gp110 (110.4) was cross-linked to Fc2, a monoclonal antibody to CD16, an antigen identified as the Fc receptor expressed on LGLs and granulocytes. LGLs are effector cells that mediate ADCC and natural killing [see, e.g., C. Ohlander et al., *Scand. J. Immunol.*, 15, pp. 409-415 (1982)]. The heteroconjugate targeted LGLs from PBL of seropositive and seronegative individuals to kill HIV-infected cells.

Thus, PBL were incubated with ^{51}Cr -labeled HIV-infected cells in the presence of a 110.4 x Fc2 heteroconjugate and lysis determined by release of ^{51}Cr . The PBL lysed the HIV-infected cells in the presence of the heteroconjugates. Less lysis was observed in control experiments where the PBL were incubated with target cells in the presence of a mere mixture of the monoclonal antibodies that make up the 110.4 x Fc2 heteroconjugate. Furthermore, as with the preceding embodiment, PBL from HIV seropositive humans were also targeted to lyse HIV-infected cells in the presence of the 110.4 x Fc2 heteroconjugate.

To determine which cells within the PBL population were actually targeted by this heteroconjugate, PBL enriched for CD8⁺ T cells and PBL enriched for CD16⁺ cells were tested for their ability to lyse HIV-infected cells. PBL enriched for CD16⁺ cells, mainly LGLs, were more cytotoxic for the HIV-infected cells than unfractionated PBL. In fact, PBL enriched for CD8⁺ T cells were less cytotoxic than the unfractionated population. Thus, CD16⁺ LGLs present within the PBL population lysed the HIV-infected cells in the presence of the 110.4 x Fc2 heteroconjugate.

The present invention also encompasses a method of treating HIV-infected individuals with pharmaceutical compositions comprising the heteroconjugates of this invention. This method of treatment may be carried out *in vivo* by the administration to an HIV-infected individual of a pharmaceutically effective amount of at least one antibody heteroconjugate of the invention. The administration of the heteroconjugate in conjunction with or after treatment with β -interferon (β -IFN), interleukin 2 (IL-2), other interferons such as α - or γ -interferon, interferon inducers or other immunomodulators may augment the effectiveness of the treatment. For example, our experiments have shown that pretreatment of PBL with β -IFN or IL-2 caused more lysis of HIV-infected cells in the presence of the heteroconjugates of this invention than the lysis observed with untreated PBL. It may also be desirable to treat HIV-infected individuals with the heteroconjugates of this invention wherein the heteroconjugates themselves are comprised of HIV-neutralizing antibodies (i.e., as the HIV-specific antibody component of the heteroconjugate) or with the heteroconjugates in conjunction with HIV-neutralizing antibodies to enhance the body's overall attack on the HIV virus.

Alternatively, the method of this invention for treating HIV-infected individuals may involve the steps of treating effector cells of the peripheral blood such as PBL with at least one antibody heteroconjugate of the invention *in vitro* and administering the effector cells and the heteroconjugate to the HIV-infected individual.

This method of treatment may also involve the *in vitro* co-incubation or preincubation of the effector cells with β -IFN or IL-2, other interferons such as α - or γ -interferon, interferon inducers or other immunomodulators, and the administration of the activated effector cells with the heteroconjugates to an HIV-infected individual. Alternatively, the effector cells may be co-incubated or preincubated *in vitro* with an antibody specific for and reactive with the particular effector cell utilized; preferably, an antibody that stimulates the lytic mechanism of the effector cell, resulting in the cell's activation. For example, when using heteroconjugates comprising antibodies to cytotoxic T cells, treatment may include co-incubation or preincubation of the effector cells with an antibody specific for T cells because of the studies that indicate that such treatment may further stimulate the lytic mechanism of cytotoxic T cells. Finally, the effector cells can also

be pretreated with a mitogen such as PHA or ConA before administration along with the heteroconjugate to the HIV-infected patient. Regardless of the method of treatment, it may be useful to use heteroconjugates comprising antibody fragments such as Fab or F(ab')₂ or chimeric antibodies.

The heteroconjugates of the invention can be administered using conventional modes of administration which include, but are not limited to, intravenous, oral, subcutaneous, intraperitoneal or intralymphatic. Intravenous administration is preferred.

The pharmaceutical compositions of the invention --comprising the heteroconjugates -- may be in a variety of dosage forms which include, but are not limited to, solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application.

The heteroconjugate compositions may include conventional pharmaceutically acceptable carriers known in the art such as serum proteins such as human serum albumin, buffer substances such as phosphates, water or salts or electrolytes.

The most effective mode of administration and dosage regimen for the heteroconjugate compositions of this invention depends upon the severity and course of the disease, the patient's health and response to treatment and the judgement of the treating physician. Accordingly, the dosages of the heteroconjugates and any accompanying compounds such as β -IFN or IL-2 should be titrated to the individual patient.

Nevertheless, an effective dose of heteroconjugate of this invention may be in the range of from about 1 to about 100 mg/m². For *in vitro* treatment of effector cells, a dose of from about 200 μ g - 2 mg of heteroconjugate/10⁹ cells administered may be used. An effective dose of β -IFN, α -IFN, or γ -IFN may be in the range of about 3X10⁶ U/patient to about 360-10⁶ U/patient with an optimum dose of 1x10⁷ U/patient. Intravenous administration is preferred when using β -IFN, whereas subcutaneous administration is preferred when using α - or γ -IFN. And, an effective dose of IL-2 may be in the range of about 1000 to about 100,000 U/kg body weight. Using a constant infusion, an effective dose may be from about 1-7 x 10⁶ U per square meter of body surface per day [see W.H. West et al., *New Eng. J. Med.*, 316 (No. 15), pp. 898-905 (1987)]. Finally, an effective dose of HIV-neutralizing antibody may be in the range of about 1 to about 100 mg/m².

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the scope of this invention in any manner.

EXAMPLE 1

The following example demonstrates the targeting of PBL from HIV seropositive and seronegative individuals by the heteroconjugates of this invention for the lysis of HIV-infected cells.

The monoclonal antibodies cross-linked to form a heteroconjugate according to this invention were an HIV-specific antibody, 110.4, and a T cell-specific, anti-CD3 antibody, G19-4. Antibody 110.4 is of subclass IgG₁, reacts with the gp110 glycoprotein of LAV within the region coded for by nucleotides 6598-7178 of LAV [see L.H. Gosting et al., *supra*], and neutralizes the infectivity of HIV. G19-4 is of subclass IgG₁ and is specific for the CD3 antigen on the T cell receptor of T lymphocytes.

Monoclonal antibody, 110.4, was prepared as follows: LAV-1 virus purified from infected CEM cells (A.T.T.C. No. CRL8904) [see F. Barre-Sinoussi et al., *Science*, 220, pp. 868-871 (1983)] was disrupted in 50 mM Tris, pH 7.4, 0.15 M NaCl, 1.0% Aprotinin, 2.0% Nonidet P-40^(R) (NP-40) (octylphenoxypolyethoxyethanol). The extract was clarified twice by centrifugation and adjusted to 0.5% NP-40 with the addition of three volumes of disruption buffer without NP-40. Lentil lectin Sepharose (Pharmacia, Piscataway, N.J.) was prewashed in disruption buffer without NP-40 and then equilibrated in adsorption buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, 1.0% Aprotinin, 0.5% NP-40). Clarified viral extract was adsorbed with lentil lectin Sepharose for 42 hours at 4° C. Unadsorbed material was removed by washing with excess adsorption buffer. Elution of adsorbed material was carried out with 0.2 M alpha methyl mannoside in adsorption buffer. The eluent was dialyzed against PBS to remove the sugar and the material was readsorbed to the lentil lectin Sepharose.

The glycoprotein-lentil lectin Sepharose complex was used to immunize BALB/c mice by three intraperitoneal injections without adjuvant given 2-3 weeks apart. Spleens were removed from immunized mice that demonstrated circulating antibody to glycoproteins of HIV by immunoblot, radioimmunoprecipitation and/or ELISA.

The procedures used for the generation of hybridoma cell lines were generally those of Kohler and

Milstein, *Nature*, 256, p. 495 (1975) with the modifications of Goldstein et al., *Infect. Immun.*, 38, p. 273 (1982). Splenic B lymphocytes from the immunized mice were fused with NS-1 myeloma cells using 40% (w/v) polyethylene glycol. Following fusion, the cell mixture was resuspended in HAT medium (RPMI-1640 medium supplemented with 15% fetal calf serum, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine) to select for the growth of hybrid cells, and then dispensed into 96-well microculture trays at a concentration of $1-3 \times 10^6$ cells/ml and incubated at 37°C in a humidified atmosphere containing 6% CO_2 . Cultures were fed by replacement of one-half the supernatant with fresh HAT medium. The wells were observed using an inverted microscope for signs of cells proliferation and when the cells were of sufficient density, the supernatants were tested for anti-LAV antibody.

Wells containing hybrid cells producing antibody to LAV were identified by ELISAs measuring the binding to either purified whole disrupted virus or biologically-expressed fusion proteins. ELISA assays using disrupted virus were carried out on LAV EIA plates (Genetic Systems, Seattle, Washington). Plates were incubated with cell culture fluids at 37°C for 45 minutes and then washed three times with 0.05% Tween 20 in phosphate-buffered saline (PBS-Tween).

Peroxidase-goat anti-mouse IgG (1:2,000 dilution in PBS-Tween; Zymed Laboratories, Inc., South San Francisco, California) was added (100 μl per well) and the plates were incubated for 45 minutes at 37°C and washed as above. Substrate (0.025 M citric acid, 0.05 M dibasic sodium phosphate, pH 5.0, containing 14 mg of o-phenylenediamine and 10 μl of 30% hydrogen peroxide per 50 ml) was added and the plates were incubated for 30 minutes at room temperature in the dark. The reaction was stopped with 3N sulfuric acid, and colorimetric reactions were quantitated with an automated microplate reader. Wells that gave positive results were subcloned by limiting dilution, retested for specificity, and then expanded.

The monoclonal antibodies secreted by the resulting hybrid cell lines were further characterized as to specificity and reactivity by immunoblotting, immunoprecipitation and ELISA using disrupted LAV virus, recombinant LAV fusion proteins and synthetic LAV peptides. All antibodies were determined to be of the IgG₁ isotype. The hybridoma that produced the 110.4 antibody utilized in this embodiment was deposited with the American Type Culture Collection under A.T.C.C. No. HB9405 in connection with commonly-owned pending U.S. patent application, Serial No. 898,273. In addition, the production of monoclonal antibodies to the gp110 and gp41 glycoproteins of HIV-1 has been described by L.H. Gosting et al., *J. Clin. Microbiol.*, 25 (No. 5), pp. 245-248 (1987). The anti-CD3 monoclonal antibody, G19-4, was prepared as described by J.A. Ledbetter and E. Clark, *Human Immunology*, 15, pp. 30-43 (1986). In addition, monoclonal antibodies to the CD3 antigen are also commercially available [see, e.g., P. Perez et al., 1986, *supra*]. The hybridoma that produces the particular anti-CD3 monoclonal antibody utilized in this embodiment of the invention, i.e., G19-4, was deposited with the American Type Culture Collection prior to the filing of this application.

The 110.4 and G19-4 monoclonal antibodies were cross-linked according to the method of B. Karpovsky et al., *supra*, using SPDP, and separated from free antibody by Sephacryl S300 size exclusion chromatography. Fractions containing high molecular weight conjugates of >300 Kd were tested in immunofluorescence assays [see, e.g., J.A. Ledbetter et al., *J. Exp. Med.*, 152, pp. 280-295 (1980)] for reactivity with a) CD3 on viable human PBL and b) acetone-fixed CEM cells that had been infected with LAV-1. Fractions with the highest binding activity to both the CD3 and the HIV antigens were then used in ^{51}Cr -release cytotoxicity assays to test the ability of PBL from HIV seropositive or seronegative individuals to lyse HIV-infected CEM cells in the presence of the 110.4 x G19-4 heteroconjugate.

The cytotoxicity assay was performed as follows: CEM cells were infected with the LAV-1 isolate for 48 hours until virtually 100% of the cells expressed gp110 as determined by indirect immunofluorescence using monoclonal antibody 110.4 followed by treatment with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G F(ab)₂ (Zymed). The infected cells were then labeled for 1 hour with ^{51}Cr ($\text{Na}_2^{51}\text{CrO}_4$, New England Nuclear, Boston, MA) and used as target cells in the assay.

The effector cells were ficoll-hypaque purified PBL from HIV seronegative or seropositive individuals. The PBL were cultured with or without monomeric anti-CD3 (G19-4) on solid phase for 3 days and then in anti-CD3-free medium for 24 hours. The untreated and treated PBL were then incubated for 4 hours at 37°C with 3×10^3 ^{51}Cr -labeled target CEM cells in 96-well microtiter plates at an effector:target cell (E:T) ratio of 50:1 with varying concentrations of the 110.4 x G19-4 heteroconjugate or mere mixtures of the individual 110.4 and G19-4 antibodies as a control. Supernatants were harvested and counted in a gamma counter. The % lysis as represented by % ^{51}Cr release was calculated as follows:

cpm experimental release - cpm spontaneous release

cpm maximal release - cpm spontaneous release

X 100

where spontaneous release = cpm released from target cells in medium alone and maximal release = cpm released from target cells in detergent. Spontaneous ^{51}Cr release was usually less than 15% of maximal release. Results shown are the mean values of % ^{51}Cr released from cells in 4 replicate wells.

Figure 1 depicts the % lysis of HIV-infected target cells by the treated and untreated PBL from seronegative individuals in the presence of the 110.4 x G19-4 heteroconjugate. As the figure indicates, the untreated PBL lysed the HIV-infected target cells in the presence of 20 to 200 ng/ml of the heteroconjugate. PBL pretreated with the anti-CD3 were even more cytotoxic than the untreated PBL.

Figure 2A depicts in table form the % lysis of HIV-infected cells vs. uninfected cells by anti-CD3-pretreated PBL from HIV seropositive and seronegative individuals in the presence of the 110.4 x G19-4 heteroconjugate or in the presence of mixtures of the individual 110.4 and G19-4 antibodies. PBL from HIV seropositive and seronegative donors were cultured with monomeric anti-CD3 (G19-4) on solid phase for 3 days and then cultured in anti-CD3-free medium for 24 hours. The PBL were then tested for cytotoxicity against HIV-infected and uninfected CEM cells at an E:T ratio of 50:1 in the presence of 200 ng/ml of the 110.4 x G19-4 heteroconjugate or a mixture of the 110.4 and G19-4 antibodies.

The data shows that a greater degree of lysis was mediated by the PBL in the presence of the heteroconjugate than in the presence of the mixture whereas there were negligible differences in the level of lysis of uninfected cells in the presence of the heteroconjugate vs. the antibody mixture. Thus, the PBL had been targeted by the heteroconjugate to kill the HIV-infected cells. The % lysis of HIV-infected or uninfected cells was not higher in the presence of the mixture of antibodies than in the absence of antibodies. Furthermore, Figure 2A indicates that PBL from asymptomatic HIV seropositive individuals are capable of lysing HIV-infected cells in the presence of the 110.4 x G19-4 heteroconjugate.

Figure 2B depicts the % lysis of HIV-infected cells by CD8⁺ enriched PBL vs. unseparated seronegative PBL in the presence of the 110.4 x G19-4 heteroconjugate. Enrichment of the PBL was performed as follows: seronegative PBL were enriched for CD8⁺ cells by negative selection as described by T. Lea et al., *Scand. J. Immunol.*, 22, pp. 207-216 (1985). Briefly, PBL, activated for 3 days with anti-CD3 on solid phase, were treated with monoclonal antibodies to DR, CD20, CD16, CD11, CD4 and CDw14 to coat B cells, monocytes, LGLs (e.g., NK or K cells) and CD4 cells. The antibody-coated cells were then incubated with magnetic particles coated with sheep anti-mouse Ig (Dynal Inc., Fort Lee, N.J.) and were removed by a Dynal M-450 magnet. All the monoclonal antibodies used -- DR (HB10a), CD20 (IF5), CD16 (Fc2.2), CD11 (60.1), CD4 (G19-2) and CDw14 (f13) have been described [see, e.g., *Leukocyte Typing*, A. Bernard et al. (ed.s), Springer-Verlag, New York (1984); *Leukocyte Typing II*, E. Reinherz et al. (ed.s), Springer-Verlag, New York (1986); and *Leukocyte Typing III*, A.J. McMichael (ed.), Oxford University Press, Oxford (in press)]. The cell separation method resulted in an approximately three-fold enrichment for CD8⁺ cells -- from 23% CD8⁺ cells in the unseparated PBL population to 62% CD8⁺ cells in the enriched population. The anti-CD3-treated unseparated and CD8⁺-enriched cells were then incubated overnight in the absence of anti-CD3 before testing for cytotoxicity with the 110.4 x CD3 heteroconjugate.

Figure 2B indicates that CD3-activated CD8⁺-enriched cells are more cytotoxic to the target cells than the unseparated cells, suggesting that the CD8⁺ cells (i.e., cytotoxic T cells) within the PBL population are largely responsible for lysing the HIV-infected cells.

This example demonstrates, therefore, the ability of the 110.4 x G19-4 heteroconjugate of this invention to target both untreated and anti-CD3-treated PBL from either HIV seropositive or seronegative individuals to lyse HIV-infected cells. The data provided clearly indicates the utility of this approach for the treatment of HIV-infected individuals.

EXAMPLE 2

This example demonstrates the ability of a heteroconjugate of the invention, comprised of monoclonal antibody 110.4 cross-linked to a monoclonal antibody specific for the Fc receptor of certain effector cells (e.g., LGLs) to target PBL to lyse HIV-infected cells.

In this example, antibody 110.4, described above, is cross-linked by the methods described above to antibody Fc2, which is specific for the CD16 antigen identified as the Fc receptor expressed on LGLs and granulocytes. Fc2 has been prepared as described by J.A. Ledbetter et al., in *Perspectives In Immunogenetics And Histocompatibility*, supra. Furthermore, the hybridoma that produces the Fc2 antibody was deposited with the American Type Culture Collection prior to the filing of this application. The resulting heteroconjugate was designated 110.4 x Fc2.

Using the same ^{51}Cr -release cytotoxicity assay described in Example 1, we tested the ability of this

heteroconjugate to target seronegative PBL that were a) cultured for 2 days with or without human IL-2 (100 U/ml, Biotest Diagnostics, Fairfield, N.J.) or b) cultured overnight with or without β -IFN (300 U/ml, HEM, Maryland) to kill HIV-infected cells. The untreated and treated PBL were incubated for 4 hours at 37°C with 3×10^3 ^{51}Cr -labeled HIV-infected CEM cells at an E:T ratio of 50:1 with varying concentrations of the heteroconjugate or an antibody mixture and % lysis determined as described in Example 1.

Figure 3 depicts the results of the assay. The PBL, both treated and untreated, were able to lyse the target cells in the presence of the heteroconjugate at heteroconjugate concentrations as low as 15 ng/ml. As shown in Figure 3A, the IL-2 activated cells were somewhat more cytotoxic toward the HIV-infected cells than the untreated PBL in the presence of the heteroconjugate. No appreciable lysis occurred in the presence of only the antibody mixture. Similarly, Figure 3B demonstrates that pretreatment of the PBL with β -IFN results in cells that are somewhat more lytic than untreated PBL in the presence of the heteroconjugate.

Figure 4 further demonstrates the ability of pretreatment with β -IFN to enhance the cytotoxicity of PBL in the presence of the 110.4 x Fc2 heteroconjugate. PBL from seronegative donors were isolated by ficoll-hypaque centrifugation, suspended at 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated human serum and β -IFN at 0, 300 or 1000 U/ml and incubated at 37°C for 3 hours. The PBL were then washed, resuspended in RPMI-1640 medium supplemented with 15% heat-inactivated fetal calf serum prior to testing for cytotoxicity against ^{51}Cr -labeled HIV-infected CEM cells. The assay was carried out as described above at an E:T cell ratio of 50:1 in a 4 hour assay in the presence of 200 ng/ml of the 110.4 x Fc2 heteroconjugate, a mixture of the individual antibodies of the heteroconjugate, the single antibodies or no antibodies. As the table in Figure 4 indicates, short term treatment of the PBL with β -IFN augments the cytotoxicity of the PBL in the presence of the heteroconjugate and thus, overnight treatment with β -IFN is not necessary.

Figure 5 depicts in table form the % lysis of HIV-infected cells by IL-2 pretreated PBL from HIV seropositive and seronegative individuals in the presence of the 110.4 x Fc2 heteroconjugate. PBL from HIV seropositive and seronegative donors were cultured for 2 days at 37°C with IL-2 (100 U/ml) and tested for cytotoxicity against HIV-infected and uninfected CEM cells at an E:T ratio of 50:1 in the presence of 200 ng/ml of the 110.4 x Fc2 heteroconjugate or a mixture of the two antibodies. This figure demonstrates the ability of PBL from seropositive (as well as seronegative) individuals to be targeted to lyse HIV-infected cells by the heteroconjugate of the invention. Augmented lysis of the uninfected cells in the presence of the heteroconjugate was not observed.

In order to determine which cells within the PBL population were responsible for the lysis seen with 110.4 x Fc2, we enriched PBL for CD16⁺ cells by the method described in Example 1, except that the monoclonal antibodies used for coating the PBL were CD28 (9.3), CD5 (10.2), CD4 (G19-2), DR (HB10a), CD20 (IF5), and CDw14 (f13), all of which have been described in the Leukocyte Typing publications cited earlier. The PBL were enriched by this method from 17% CD16⁺ cells in the unseparated PBL population to 66% CD16⁺ cells in the enriched population. The unseparated and CD16⁺-enriched cells were cultured for 2 days with IL-2 prior to testing for cytotoxicity at various E:T ratios in the presence of 200 ng/ml of 110.4 x Fc2. As Figure 6 indicates, the IL-2-treated PBL enriched for CD16⁺ cells were more cytotoxic for HIV-infected cells than the unseparated cells, suggesting that CD16⁺ LGL cells within the PBL population are targeted by this heteroconjugate for the lysis of the HIV-infected cells.

This example therefore demonstrates the ability of the heteroconjugates of this invention to target a second effector cell of the peripheral blood, i.e., LGL cells, for the killing of HIV-infected cells. In addition, we have demonstrated that pretreatment of the effector cells with either IL-2 or β -IFN enhances the ability of the heteroconjugates to target the effector cell to lyse the HIV-infected cell.

EXAMPLE 3

This example demonstrates the ability of another heteroconjugate of the invention, comprising a monoclonal antibody to a second HIV glycoprotein, gp41, cross-linked to monoclonal antibody, G19-4, to target PBL to lyse HIV-infected cells.

In this example, we utilized monoclonal antibody 41.1 as the HIV-specific antibody of the heteroconjugate. 41.1 is an antibody of subclass IgG₁ and reacts with an epitope on a highly conserved region of gp41 encoded by nucleotides 7178-7698 of LAV-1. The production of this monoclonal antibody is described in detail in L.H. Gosting et al., *supra*, and the hybridoma that produces the antibody has been deposited with the American Type Culture Collection prior to the filing of this application.

41.1 and G19-4 were cross-linked as described in Example 1 and the resulting heteroconjugate was designated 41.1 x G19-4. As in Example 1, the heteroconjugate was separated from free antibody by size exclusion chromatography using Sephacryl S300 and the fraction with the highest binding activity to both gp41 on HIV-infected cells and to CD3 antigen on human PBL was then tested in the cytotoxicity assay described in Example 1. Briefly, the effector cells were PBL from seronegative donors that had been cultured for three days with anti-CD3 on solid phase followed by overnight incubation in anti-CD3-free medium. The effector cells were then incubated for 4 hours at 37°C with 3×10^3 ^{51}Cr -labeled target CEM cells as described in Example 1 at a E:T ratio of 50:1 in the presence of either a) the 41.1 x G19-4 heteroconjugate; b) a 41.1 plus G19-4 antibody mixture; c) 41.1 alone or d) G19-4 alone and % lysis of the CEM cells determined. The results of this assay are depicted in Figure 7. The figure indicates the ability of the 41.1 x G19-4 heteroconjugate to target PBL to kill HIV-infected cells. The PBL were stimulated to lyse the HIV-infected cells in the presence of approximately 50 to 200 ng/ml of the heteroconjugate. Lysis in the presence of the antibody mixture or the individual antibodies alone was negligible.

Thus, the heteroconjugate approach of this invention for killing HIV-infected cells can employ any of a number of HIV-specific antibodies cross-linked to effector cell-specific antibodies. In fact, the 41.1 monoclonal antibody is particularly useful in this approach because it was reactive with a majority of the isolates of HIV tested (10 out of 13), the isolates having been derived from various geographical areas of the world.

Hybridomas prepared by the processes described herein are exemplified by cultures deposited in the American Type Culture Collection, Rockville, Maryland. The cultures were deposited on September 15, 1987, and are there identified as follows:

Hybridoma G19-4: ATCC No. HB9536

Hybridoma Fc2: ATCC No. HB9535

Hybridoma 41.1: ATCC No. HB9534.

While we have hereinbefore presented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the methods and heteroconjugates of this invention. For example, other monoclonal antibodies to other HIV antigens such as "gag" antigens or to other antigens on T cells, LGLs or other effector cells in the blood may be utilized to construct the heteroconjugates and perform the methods of the invention. Therefore, it should be understood that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

Claims

1. An antibody heteroconjugate comprising at least one antibody reactive with an HIV antigen expressed on the surface of an HIV-infected cell cross-linked to at least one antibody reactive with an effector cell of the peripheral blood capable of killing an HIV-infected cell.
2. The antibody heteroconjugate of claim 1, wherein the HIV antigen is one found on an HIV envelope glycoprotein.
3. The antibody heteroconjugate of claim 1, wherein the effector cell is selected from the group consisting of T lymphocytes, large granular lymphocytes, granulocytes, monocytes and macrophages.
4. The antibody heteroconjugate of claim 1, wherein the effector cell-reactive antibody is selected from the group consisting of antibodies reactive with the T cell receptor on T lymphocytes and an Fc receptor on leukocytes.
5. The antibody heteroconjugate of claim 1, wherein the effector cell-reactive antibody is an antibody to the CD3 antigen on the T cell receptor of T lymphocytes.
6. The antibody heteroconjugate of claim 1, wherein the effector cell-reactive antibody is an antibody to the CD16 Fc receptor of large granular lymphocytes and granulocytes.
7. The antibody heteroconjugate of claim 1, wherein the antibodies are antibody fragments selected from the group consisting of Fab and F(ab)_2 fragments.
8. The antibody heteroconjugate of claim 1, wherein the antibodies are chimeric antibodies.
9. An antibody heteroconjugate comprising a first antibody reactive with an HIV antigen expressed on the surface of an HIV-infected cell cross-linked to a second antibody reactive with an effector cell of the peripheral blood capable of killing an HIV-infected cell.
10. Antibody heteroconjugates selected from the group consisting of 110.4 x G19-4, 110.4 x Fc2 and 41.1 x G19-4.

11. The use of effector cells of the peripheral blood in the presence of at least one antibody heteroconjugate according to claim 1 for preparing a pharmaceutical composition for killing HIV infected cells.

12. The use of claim 11, wherein the effector cells are selected from the group consisting of peripheral blood lymphocytes, granulocytes, monocytes and macrophages.

13. The use of claim 11, wherein the effector cells are obtained from HIV seropositive or seronegative individuals.

14. The use of claim 11, wherein the effector cells are pretreated with a compound selected from the group consisting of interleukin-2, β -interferon, α -interferon and γ -interferon.

15. The use of claim 11, wherein the effector cells are pretreated with an antibody specific for the effector cells.

16. The use of claim 15, wherein the antibody is one that stimulates the lytic mechanism of the effector cells.

17. The use of claim 16, wherein the antibody is an anti-CD3 antibody.

18. The use of claim 11 or 14, wherein the effector cells are cytotoxic T lymphocytes and the antibody heteroconjugate is selected from the group consisting of 110.4 x G19-4 and 41.1 x G19-4.

19. The use of claim 11 or 14, wherein the effector cells are large granular lymphocytes and the antibody heteroconjugate is 110.4 x Fc2.

20. A pharmaceutically acceptable composition useful in the treatment of HIV infections which comprises a pharmaceutically effective amount of at least one antibody heteroconjugate according to claim 1.

21. The use of a pharmaceutically effective amount of at least one antibody heteroconjugate according to anyone of claims 1 to 10 for preparing a pharmaceutical composition for treating HIV infections.

22. The use of claim 21, wherein additionally a pharmaceutically effective amount of a compound selected from the group consisting of interleukin-2, β -interferon, α -interferon and γ -interferon is employed.

23. The use of effector cells of the peripheral blood capable of killing HIV-infected cells which effector cells were treated with at least one antibody heteroconjugate according to anyone of claims 1 to 10 in vitro and of said heteroconjugate for preparing a pharmaceutical composition for treating HIV infections.

24. The use of claim 23, wherein the effector cell is selected from the group consisting of T lymphocytes, large granular lymphocytes, granulocytes, monocytes and macrophages.

25. The use of claim 23, wherein the effector cells are pretreated with a compound selected from the group consisting of interleukin-2, β -interferon, α -interferon and γ -interferon.

26. The use of claim 23, wherein the effector cells are pretreated with an antibody specific for the effector cells.

27. The use of claim 26, wherein the antibody is one that stimulates the lytic mechanism of the effector cell.

28. The use of claim 27, wherein the antibody is an anti-CD3 antibody.

29. A process for preparing an antibody heteroconjugate having at least one first antibody reactive with an HIV antigen expressed on the surface of an HIV-infected cell cross-linked to at least one second antibody reactive with an effector cell of the peripheral blood capable of killing an HIV-infected cell, comprising the step of reacting said first and second antibody with a heterobifunctional cross-linking agent.

30. The process of claim 1, wherein the cross-linking agent is SPDP or GMBS.

31. The process of claim 1, wherein said first antibody is 110.4 and said second antibody is G19-4; said first antibody is 110.4 and said second antibody is Fc2; or said first antibody is 41.1 and said second antibody is G19-4.

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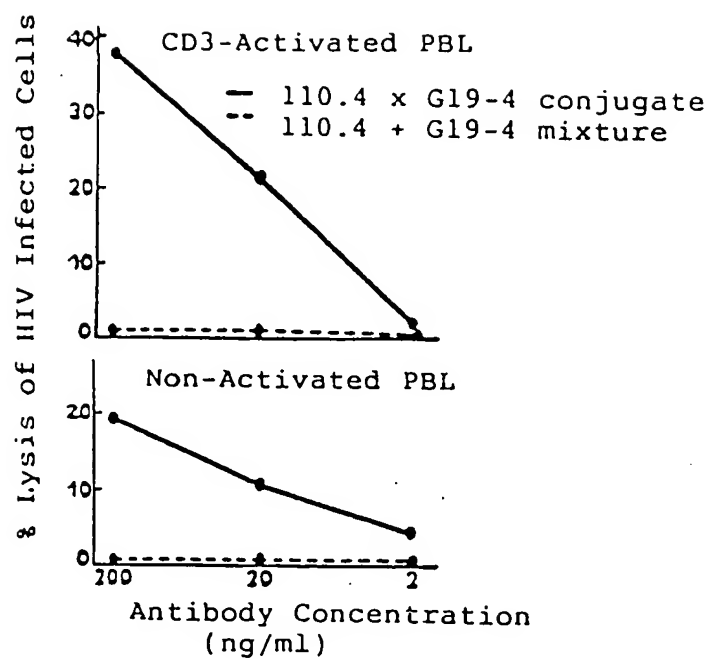
FIGURE 1

FIGURE 2

A. Experiment Donor	% lysis of HIV-infected cells		% lysis of uninfected cells	
	110.4xG19-4 conjugate	110.4+G19-4 mixture	110.4xG19-4 conjugate	110.4+G19-4 mixture
1. A (sero-)	42.9	14.5	3.5	9.5
B (sero+)	88.4	25.2	14.0	14.6
2. C (sero-)	41.9	11.9	21.3	18.3
D (sero-)	27.7	8.8	15.1	13.6
E (sero+)	49.3	19.9	8.0	12.0
F (sero+)	22.5	4.4	2.7	8.7
3. G (sero-)	23.5	-2.4	NT	NT
H (sero-)	20.7	3.4	NT	NT

B. CD3-activated Effector Cells	% lysis of HIV-infected cells	
	E:T 50:1	12.5:1
Unseparated sero- cells	24.3	6.1
CD8 ⁺ enriched cells	36.0	24.6

FIGURE 3

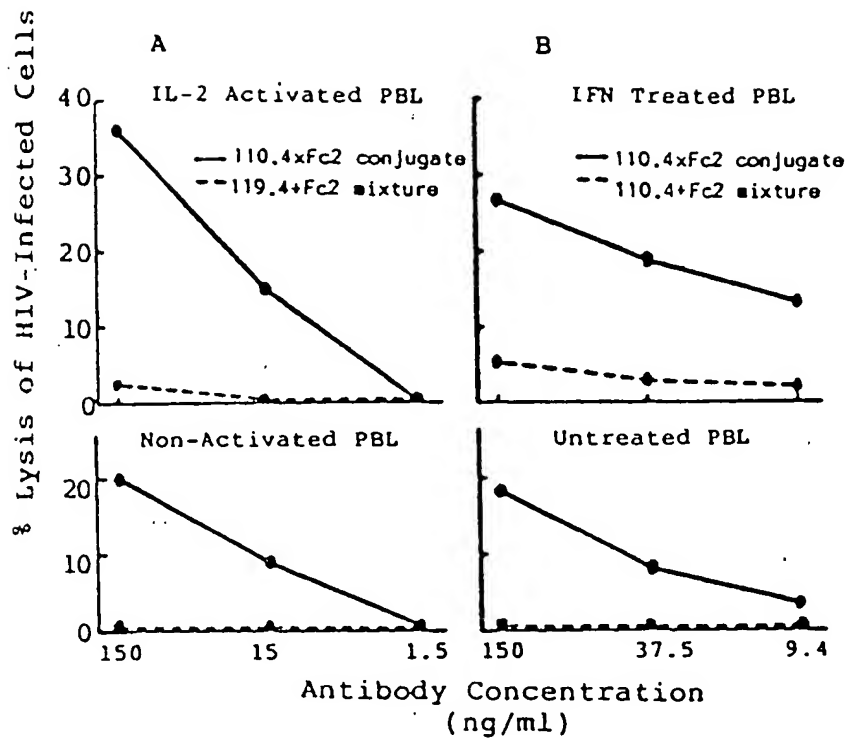


FIGURE 4% LYSIS OF HIV-INFECTED CELLS

<u>Antibody (200ng/ml)</u>	Donor 366			Donor 372		
	PBL treated with			PBL treated with		
	<u>IFN-B (U/ml)</u>			<u>IFN-B (U/ml)</u>		
	<u>1000</u>	<u>300</u>	<u>0</u>	<u>1000</u>	<u>300</u>	<u>0</u>
110.4xFc2 conjugate	41.3	26.9	18.7	37.6	44.0	27.4
110.4 alone	9.7	-	7.1	23.4	16.4	12.3
Fc2 alone	2.1	7.3	2.4	18.6	7.9	9.7
110.4+Fc2 mixture	11.6	7.3	3.1	18.0	11.1	12.7
None	10.2	5.1	2.4	15.0	8.0	5.8

FIGURE 5

Experiment	Donor	<u>% lysis of HIV-infected cells</u>		<u>% lysis of uninfected cells</u>	
		110.4 x Fc2 conjugate	110.4 + Fc2 mixture	110.4 x Fc2 conjugate	110.4 + Fc2 mixture
1	A (sero-)	31.8	11.6	12.5	10.3
	G (sero-)	50.3	22.7	14.2	18.2
	B (sero+)	28.9	8.6	8.2	6.1
2	C (sero-)	60.2	28.4		
	D (sero-)	35.5	18.3		
	E (sero+)	38.0	17.5		
	F (sero+)	30.1	8.6		

FIGURE 6

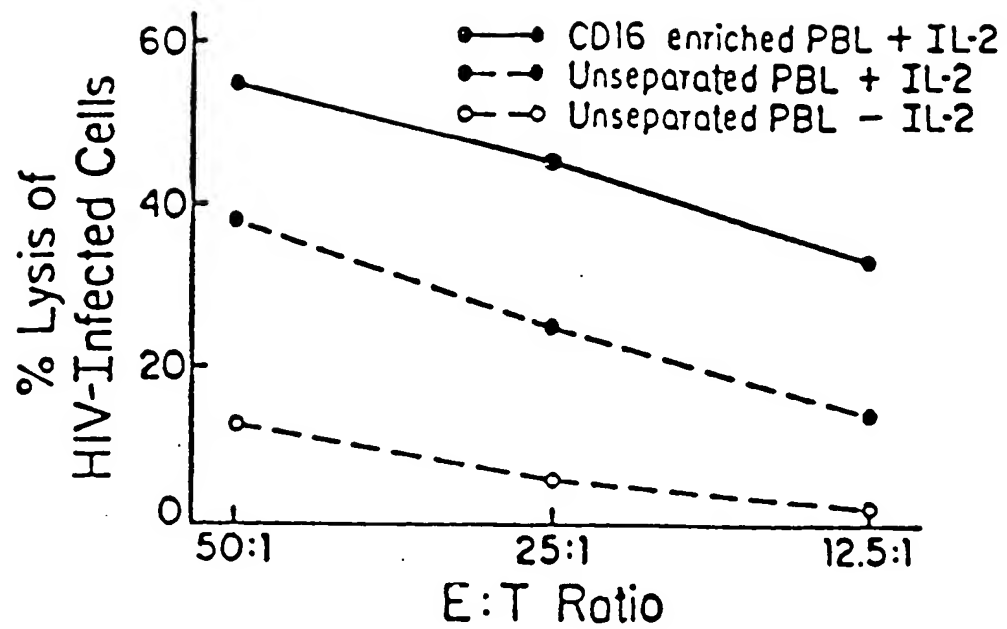


FIGURE 7% LYSIS OF HIV-INFECTED CELLS

	Donor 372 Antibody <u>Concentration (ng/ml)</u>	
	<u>200</u>	<u>50</u>
41.1xG19-4 conjugate	23.0	15.4
41.1+G19-4 mixture	1.9	3.7
41.1 alone	3.9	3.3
G19-4 alone	-0.9	1.3